Acid-induced Lung Injury

Protective Effect of Anti-interleukin-8 Pretreatment on Alveolar Epithelial Barrier Function in Rabbits

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Although prior experimental work has demonstrated that anti-interleukin-8 (anti-IL-8) therapy reduces lung endothelial injury after acid instillation, there is no information regarding the effect of anti-IL-8 on the function of the alveolar epithelial barrier after acid-induced lung injury. Therefore, the primary objective of this study was to determine the effect of acid-induced lung injury on the function of the alveolar epithelium, and secondly to determine whether pretreatment with anti-IL-8 attenuates acid-induced injury to the lung epithelial barrier. Hydrochloric acid (pH = 1.5 in 1/3 normal saline) was instilled into the lungs of anesthetized, ventilated rabbits. Anti-IL-8 monoclonal antibody (2 mg/kg) or saline was given intravenously 5 min before acid instillation. Acid instillation into the distal airspaces caused an increase in the alveolar epithelial permeability to protein and an approximately 50% reduction in net alveolar fluid clearance. Because a decrease in net alveolar fluid clearance could be due to lung endothelial injury and increased fluid flux from the blood into the airspaces, additional experiments were carried out in which pulmonary blood flow was eliminated. In the absence of pulmonary blood flow, acid instillation led to a 50% decrease in net alveolar fluid clearance. Pretreatment with anti-IL-8 antibody significantly reduced the acid-mediated increase in bi-directional transport of protein across the alveolar epithelium and restored alveolar fluid clearance to normal. The results indicate that acid instillation causes injury to the alveolar epithelial barrier that can be distinguished from the injury to the lung endothelium. Furthermore, pretreatment with anti-IL-8 therapy prevents acid-induced alveolar epithelial injury, a finding of potential clinical importance. **Modelska K, Pittet JF, Folkesson HG, Broaddus VC, Matthay MA. Acid-induced lung injury: protective effect of anti-interleukin-8 pretreatment on alveolar epithelial barrier function in rabbits.**

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Acid aspiration–induced lung injury is an important cause of the acute respiratory distress syndrome (ARDS) with a high mortality rate (1, 2). The effect of acid instillation on the pulmonary endothelial barrier has been extensively studied (3–8). We, and other investigators, have reported that acid aspiration–induced lung injury is mediated primarily by neutrophils recruited to the lung by interleukin-8 (IL-8) (7, 9–12). In clinical studies, higher IL-8 concentrations in pulmonary edema and bronchoalveolar lavage fluid from patients with acute lung injury were associated with a trend toward higher mortality (13, 14). In experimental studies, anti-rabbit-IL-8 monoclonal antibody (anti-IL-8) attenuated acid-induced lung injury and prevented acid-induced abnormalities in oxygenation, extravascular lung water formation, and lung endothelial permeability (7). However, there is little direct information on the function of the alveolar epithelial barrier after acid-induced injury.

Because experimental (15–18) and clinical (19) studies indicate that the function of the alveolar epithelial barrier is essential for successful recovery from acute lung injury, we wanted to test the effect of anti-IL-8 pretreatment on the function of the alveolar epithelium directly.

Therefore, the first objective of these studies was to determine the effect of acid-induced lung injury on the barrier properties and the fluid transport capacity of the alveolar epithelium. The second objective was to test whether the injury to the epithelial barrier was mediated by IL-8. Therefore, rabbits were pretreated with anti-IL-8 to determine the effect on alveolar epithelial fluid transport capacity and alveolar epithelial permeability to protein after acid-induced lung injury.

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METHODS

Animals, Surgical Preparations, and Ventilation

Male New Zealand white rabbits (n = 31, weighing 2.5 to 3.5 kg; Nitabell, Hayward, CA) were anesthetized with 0.8% halothane in 100% oxygen. Pancuronium bromide (Pavulon; Organon Diagnostic, West Orange, NJ; 0.3 mg/h × kg body weight) was given intravenously for neuromuscular blockade. The University of California, San Francisco

EXHIBIT B

(UCSF) Animal Research Committee approved the protocol for these studies.

A 4.0-mm inside diameter endotracheal tube was inserted through a tracheotomy. The rabbits were ventilated with a constant-volume piston pump (Harvard Apparatus Co., South Natick, MA) with an inspired oxygen fraction of 1.0, peak airway pressures of 15 to 18 cm H_2O during the baseline period, supplemented with positive end-expiratory pressure of 4 cm H_2O. The respiratory rate was adjusted to maintain the Pa_{CO_2} between 35 and 40 mm Hg during the baseline period. The ventilator settings were then kept constant throughout the experiments. A PE-90 catheter (Clay Adams; Becton Dickinson, Parsippany, NJ) was inserted into the right carotid artery to monitor systemic arterial blood pressure and to obtain blood samples. A 22-gauge angiocath catheter (Deseret Medical, Inc., Becton Dickinson, Parsippany, NJ) was inserted into the marginal ear vein for administering fluid and drugs. After the surgical preparation, the rabbits were placed in the right lateral decubitus position.

Preparation of the Instillate

A solution of 100 mosmol/kg of NaCl (1/3 normal saline) was prepared with isotonic 0.9% saline and distilled water. This osmolality is approximately one-third of plasma osmolality. The 1/3 normal osmolality was selected to match the osmolality of gastric aspirates, as we have done before (7). Then, concentrated HCl was added to the solution and titrated to a pH of 1.5. In negative control studies, 1/3 normal saline was used as the instillate. The HCl and the control solutions had similar measured osmolalities.

Generation of Monoclonal Antibodies to Rabbit rIL-8

The generation of the monoclonal antibodies to rabbit recombinant IL-8 (rIL-8) (ARIL8.2) has been described in detail earlier (7, 20). ARIL8.2 was selected by virtue of its ability to recognize rabbit IL-8, to inhibit binding of ^{125}I-labeled rabbit rIL-8 to its receptor, to block rabbit rIL-8-induced receptor signal transduction, and to inhibit rabbit rIL-8-induced chemotactic activity for rabbit neutrophils (20). ARIL8.2 had a high affinity for rabbit IL-8 (dissociation constant [K_d] = 0.42 nM). ARIL8.2 did cross-react with human IL-8, but not with closely related cytokines (hMGSA, platelet factor-4, β-thromboglobulin), other human cytokines (IL-1β, tumor necrosis factor-alpha [TNF-α]), or other chemotactic factors (formylmethylleucylphenylalanine [FMLP], C5a). The antibody preparation was sterile filtered and endotoxin was undetectable by *Limulus* assay.

General Experimental Protocol

In all experiments (Figure 1), after surgery, heart rate, systemic blood pressure, and arterial blood gases were allowed to stabilize for 60 min in pretreatment experiments. After the baseline, anti-IL-8 (2 mg/kg) or saline was given intravenously 5 min before HCl was instilled through a 5-French tubing (Accumark Premarked Feeding Catheter; Concord/Portex, Keene, NH), inserted into the lower lobe of the right lung (Figure 2). This tubing remained in this position for the duration of the experiment; the same tubing was subsequently used for alveolar fluid instillation to ensure that the 5% albumin solution was instilled in the same lung lobe as the acid. The tubing was also used for sampling the alveolar fluid at 5 min and 2 h after instillation in each experiment (Figure 1).

HCl or 1/3 normal saline (4 ml/kg body weight) was instilled into the right lung over 3 min. Then, 210 min after the HCl instillation (Figure 1), a vascular tracer, ^{131}I-labeled human albumin (1 μCi; Frosst Laboratories, PQ, Canada), was injected into the blood to provide a vascular protein tracer that could be used to measure accumulation of plasma protein into the extravascular space, as we have before (7). Blood samples were obtained at 30 and 45 min after the injection of vascular tracer. Then, 45 min after the vascular tracer injection, 3 ml/kg of 5% bovine albumin solution containing the alveolar tracer, ^{125}I-albumin (1 μCi; Frosst Laboratories) was instilled in the 5% albumin solution with a 12-ml syringe into the right lower lobe through the same catheter previously used for the HCl instillation (Figure 2). The alveolar tracer was used to calculate the flux of labeled protein from the airspaces into the circulating plasma. Blood samples were obtained at 60 and 120 min after instillation of the alveolar tracer. In addition, samples from the alveolar fluid were obtained at 5 and 120 min after alveolar fluid instillation. These samples were used for radioactivity counts and protein measurements.

In the pretreatment experiments, the anti-IL-8 monoclonal antibody was given 5 min before HCl instillation. In experiments without blood flow, to eliminate vascular filtration to the right lung, the right pulmonary artery was occluded with a suture 210 min after acid instillation and 10 min before the injection of vascular tracer (Figure 1).

At the end of the 6-h experiment (2 h after the beginning of alveolar fluid and tracer instillation), the abdomen was opened and the rabbits were exsanguinated. Urine was collected for radioactivity counts. The lungs were removed through a median sternotomy. The left, non-instilled lung was clamped at the main bronchus. Then, an alveolar fluid sample was obtained from the right lower lobe of the instilled lung. The right and the left lungs were subsequently homogenized separately for water-to-dry weight ratio measurements and radioactivity counts.

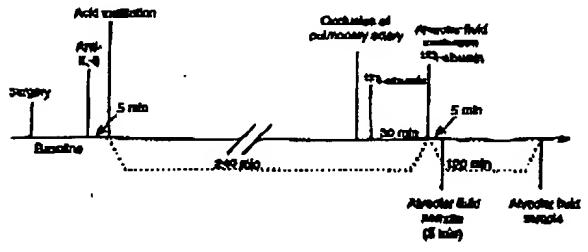

Figure 1. General experimental protocol as described in detail in METHODS.

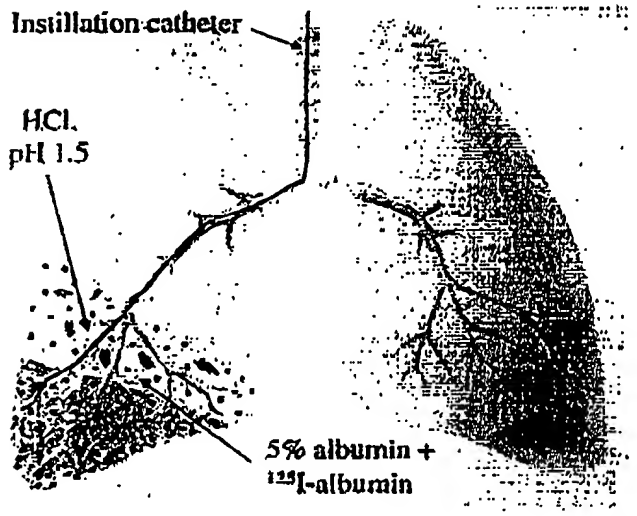

Figure 2. Schematic diagram illustrating the location of the 5-French catheter for acid or saline instillation as well as for obtaining alveolar fluid samples. The right pulmonary artery was occluded in studies without vascular filtration. The denser shading of the distal lung segment illustrates that there was a lung region in which there was dense homogenous blue labeled accumulation of the alveolar instillate, whereas the less shaded, more proximal lung segment designates heterogenous filling of the airspaces. The *sharp black dots* refer to the ^{125}I-labeled albumin in the instillate, and the *irregular shaped dots* designate the 5% unlabeled albumin in the instillate.

Specific Experimental Protocol

There were six experimental groups. The rabbits from the first three groups had intact pulmonary blood flow and the rabbits from the last three groups had the right pulmonary artery occluded.

Studies with Intact Pulmonary Blood Flow

Negative control group (1/3 normal saline) (n = 5). After the baseline period, the saline was instilled into the right lower lobe over 3 min. Alveolar fluid was instilled after 4 h. The rabbits were studied for a total time of 6 h and processed as described previously in the GENERAL EXPERIMENTAL PROTOCOL. We did not study rabbits with an irrelevant monoclonal antibody because the results from previous studies from our laboratory indicated that there were no differences in extravascular plasma equivalents nor in the extravascular lung water between rabbits given 0.9% NaCl or an irrelevant monoclonal antibody (7).

HCl instillation (positive control group) (n = 6). After the baseline period, HCl (4 ml/kg body weight) was instilled into the right lower lobe over 3 min. Alveolar fluid was instilled after 4 h. The rabbits were processed as described in the GENERAL EXPERIMENTAL PROTOCOL.

Anti-IL-8 pretreatment group (n = 6). Five min before the HCl instillation, the rabbits received the monoclonal antibody against IL-8 (ARIL8.2, 2 mg/kg body weight) intravenously. Then, HCl (4 ml/kg body weight) was instilled into the right lower lobe over 3 min. Alveolar fluid was instilled after 4 h. The rabbits were studied for a total time of 6 h and processed as described in the GENERAL EXPERIMENTAL PROTOCOL.

Studies without Pulmonary Blood Flow

Negative control group (1/3 normal saline) (n = 5). After the stable baseline period, saline was instilled into the right lower lobe with 1/3 normal saline (4 ml/kg body weight) over 3 min. Then, 210 min later the right pulmonary artery was occluded. Alveolar fluid was instilled into the lung without vascular filtration for the last 2 h of each experiment and the rabbits were processed as described in the GENERAL EXPERIMENTAL PROTOCOL.

HCl instillation (positive control group) (n = 6). After the stable baseline period, HCl was instilled (4 ml/kg body weight) over 3 min and then, 210 min later the right pulmonary artery was occluded. Alveolar fluid was instilled into the lung without vascular filtration for the last 2 h of each experiment and the rabbits were processed as described in the GENERAL EXPERIMENTAL PROTOCOL.

Anti-IL-8 pretreatment group (n = 6). The rabbits were pretreated with the monoclonal antibody against IL-8 (ARIL8.2, 2 mg/kg body weight) intravenously, 5 min before the HCl instillation. Then, HCl was instilled over 3 min, and 210 min later the right pulmonary artery was occluded. Alveolar fluid was instilled into the lung without vascular filtration for the last 2 h of each experiment and the rabbits were processed as described in the GENERAL EXPERIMENTAL PROTOCOL.

Measurements

Hemodynamics, pulmonary gas exchange, and protein concentration. The heart rate, systemic blood pressure, and airway pressures were continuously measured using calibrated pressure transducers (Pd23; Gould, Inc., Oxnard, CA) and recorded on a polygraph (Model 7; Grass Instrument Co., Quincy, MA). Arterial blood gases and pH were measured at 1-h intervals. Samples from alveolar fluid instillation, from the final alveolar fluid samples, and from the initial and final blood were collected to measure total protein concentration.

Protein concentration and hemoglobin measurement. Protein concentration was measured by the Biuret method. Hemoglobin was measured spectrophotometrically on the last blood sample and on the supernatant obtained after centrifugation of the lung homogenate (14,000 × *g* for 10 min).

Alveolar liquid clearance. The alveolar liquid clearance was measured by the increase in the final unlabeled alveolar protein concentration compared with the initial alveolar protein concentration, as we have done in earlier studies (21). Alveolar liquid clearance (ALC) was then calculated as: $ALC\ (\%) = (V_i \times Fw_i - V_f \times Fw_f)/(V_i \times Fw_i) \times 100$, where Fw is the water fraction of the initial (i) and final (f) alveolar fluid. The water fraction is the volume of water per volume of solution measured by the gravimetric method. V is the volume of the initial (i) and final (f) alveolar fluid. V_f (ml) was estimated as: $V_f = (V_i \times TP_i \times Fr)/TP_f$, where TP is the total protein concentration of the initial (i) and final (f) alveolar fluid. Fr is the fraction of alveolar tracer (^{125}I-albumin) protein that remained in the lung at the end of the experiment. The radioactivity of the alveolar ^{125}I-albumin can also be used to estimate alveolar liquid clearance (21). The volume of the final alveolar fluid was then estimated as: $V_f = (V_i \times {}^{125}cpm_i \times Fr)/{}^{125}cpm_f$, in which cpm refers to counts per min of the initial and the final sample after 2 h.

Because alveolar liquid clearance was measured in the presence of lung injury, we used our prior method (22, 23) to correct for both the filtration and the loss of the alveolar protein tracer (^{125}I-albumin) indicator. Because some edema fluid was present in the alveoli in rabbits 4 h after acid instillation, we used the dilution of the instilled ^{125}I-albumin solution sampled at 5 min after instillation (Figure 1) to calculate alveolar liquid clearance (22, 23). The instilled alveolar ^{125}I-albumin concentration was diluted on average to 85% of the instilled concentration in the 5-min samples from the rabbits that had been instilled with acid (*see* RESULTS). Therefore, alveolar liquid clearance was calculated by the rise in the ^{125}I-albumin concentration over 2 h, beginning with the concentration in duplicate samples taken 5 min after instillation. Because some of the alveolar protein tracer was lost from the alveolar spaces over the 2 h of the alveolar fluid clearance studies (Table 1), we also adjusted for the loss of the alveolar tracer (^{125}I-albumin) into the plasma. Thus, the instilled concentration of ^{125}I-albumin was adjusted by both the dilution after 5 min (85% of the instilled) and by the 10.7% of the alveolar tracer that appeared in the plasma over 2 h. It is possible that a small fraction of the ^{125}I-albumin may be in the lung interstitium and thus not accounted for by this method.

For example, if the instilled ^{125}I-albumin counts were 100,000 cpm/g, then 85% dilution would mean that the actual counts after instillation were 85,000 cpm/g. If 10.7% of the instilled solution appeared in the plasma over 2 h, then another 10,700 cpm/g was subtracted so that the

TABLE 1

EFFECT OF ACID INSTILLATION ON BIDIRECTIONAL PROTEIN PERMEABILITY ACROSS THE LUNG ENDOTHELIAL AND EPITHELIAL BARRIERS IN RABBITS*

Experimental Condition	n	Alveolar Protein Tracer ^{125}I-albumin (% of instilled)		Vascular Protein Tracer ^{131}I-albumin
		Lung	Plasma	(Alv/plasma ratio)
With pulmonary blood flow				
Saline (negative controls)	5	97.8 ± 0.7	1.8 ± 0.6	0.01 ± 0.00
HCl (positive controls)	6	87.9 ± 2.8†	10.8 ± 3.2†	0.45 ± 0.09†
Anti-IL-8 + HCl	6	94.1 ± 1.3‡	4.1 ± 1.4‡	0.05 ± 0.01‡
Without pulmonary blood flow				
Saline	4	99.7 ± 0.7†	0.5 ± 0.2†	0.05 ± 0.02
HCl	6	93.2 ± 1.1	3.5 ± 0.6	0.04 ± 0.01
Anti-IL-8 + HCl	4	99.5 ± 0.3‡	0.1 ± 0.1‡	0.09 ± 0.01

* Data are mean ± SEM.
† $p < 0.05$ from saline-instilled rabbits.
‡ $p < 0.05$ from HCl-instilled rabbits.

net quantity of the alveolar tracer present in the alveoli after 2 h would be 85,000 cpm/g − 10,700 cpm/g which equals 74,300 cpm/g. If the measured final alveolar sample after 2 h had 90,000 cpm/g, then the final to instilled (corrected) counts would be 90,000/74,300. Alveolar liquid clearance could then be calculated as described previously.

Albumin flux across endothelial and epithelial barriers. Two different methods were used to measure the flux of albumin across the lung endothelial and epithelial barriers, as we have done before (16, 22). The first method measures residual ^{125}I-albumin (the airspace protein tracer) in the lungs as well as accumulation of ^{125}I-albumin in plasma. The second method measures ^{131}I-albumin (the vascular protein tracer) in the extravascular and alveolar spaces of the lungs.

The total quantity of ^{125}I-labeled albumin instilled into the lung was determined by measuring the radioactivity of duplicate samples of the instilled solution (cpm/g) and multiplying by the total volume instilled into the lung. To calculate residual ^{125}I-albumin in the lungs at the end of the study, the average radioactivity of duplicate samples obtained from the lung homogenate was multiplied by the total weight of lung homogenate. The ^{125}I-albumin counts in the lung homogenate data were added to the recovered counts in the final aspirated distal airspace fluid to calculate the quantity of instilled ^{125}I-albumin that remained in the lungs at the end of the study. The ^{125}I-albumin was measured in plasma from the final blood sample. The ^{125}I-albumin in the aspirates was measured from a sample obtained 5 min after the start of alveolar fluid instillation and at the end of the experiment. The ^{125}I-albumin was measured in plasma from the final blood sample. The amount of ^{125}I-albumin in plasma was accounted for by multiplying the cpm/g by the plasma volume (body weight × 0.07 [1-hematocrit]) (7).

The second method requires measurement of the vascular protein tracer, ^{131}I-albumin, in the alveolar and extravascular spaces of the lungs. To calculate the amount of ^{131}I-albumin present in the extravascular spaces of the lung, we deducted the counts of the blood in the lung from the ^{131}I-albumin counts in the entire lung. The clearance of plasma into the extravascular spaces of the lung was estimated by the following equation and expressed as extravascular plasma equivalents (EPE): $EPE = {}^{131}cpm/ml_{lung} - ({}^{131}cpm/ml_{Pf} \times Q_b)/{}^{131}cpm/ml_{Pf}$, in which $^{131}cpm_{lung}$ is the total counts per min in lung, $^{131}cpm_{Pf}$ is the cpm per milliliter of plasma in the final blood sample, $^{131}cpm_{Pf}$ the cpm per milliliter plasma averaged over the time of the experiment, and Q_b is the blood volume in the lung.

The blood volume was determined from the equation: $Q_b = 1.039 \times (Q_h \times FW_h \times Hb_s)/(FW_s \times Hb_B)$, where 1.039 is the density of blood, Q_h the weight of lung homogenate, FW_h the water content of lung homogenate, Hb_s the hemoglobin concentration of supernatant of lung homogenate, FW_s the water content in supernatant of lung homogenate, and Hb_B the hemoglobin concentration of blood.

A ratio between the ^{131}I-albumin counts in the final alveolar fluid sample, and ^{131}I-albumin plasma counts provided an index of equilibration of the vascular protein tracer into the alveolar compartment, as in earlier experimental studies of epithelial permeability (16).

Tracer binding measurement. To determine ^{125}I and ^{131}I stability and binding to albumin, trichloroacetic acid (20%) was added to selected samples, which were centrifuged to obtain the supernatant for measurement of free ^{125}I radioactivity. The results are expressed as a percentage of the unbound ^{125}I radioactivity to the total amount of ^{125}I-albumin radioactivity instilled. These samples had always less than 1% unbound iodine.

Statistics

All data in the figures are summarized as mean ± 1 SD; the data on radioactivity in Table 1 are presented as mean ± SEM. One-way ANOVA with repeated measurement analysis was used to compare samples obtained at several time points from the same animal. One-way analysis of variance and the Fisher exact *t* tests were used to compare experimental with control groups. A p value of < 0.05 was considered statistically significant.

RESULTS

Studies with Pulmonary Blood Flow

Effects of acid instillation on alveolar epithelial permeability to protein. To simulate acid-aspiration acute lung injury, 12 ± 2 ml of hydrochloric acid (HCl, pH = 1.5 in 1/3 normal saline) were rapidly instilled into the right lower lobe (Figure 2). Acid instillation caused a bi-directional increase in alveolar epithelial permeability to protein (Table 1). There was a significant increase in the protein efflux (alveolar protein tracer, ^{125}I-albumin) from the airspaces to the plasma in the acid-instilled rabbits compared with the saline-instilled rabbits (Table 1). In addition, there was a significant increase in the protein influx (vascular protein tracer, ^{131}I-albumin) from the plasma into the airspaces. The alveolar-to-plasma concentration ratio of ^{131}I-albumin was significantly increased in acid-instilled rabbits compared with the saline-instilled rabbits (Table 1).

Effects of acid instillation on fluid transport across the alveolar barrier. To measure the dilution of the instilled labeled and unlabeled protein solution by the presence of protein-rich pulmonary edema, samples were obtained 5 min after instillation (Figure 1). The initial unlabeled alveolar protein concentration was not different from the protein concentration of the instilled 5% albumin solution. In contrast, the concentration of ^{125}I-albumin was decreased in the initial alveolar sample compared with the instilled albumin solution (initial alveolar [after 5 min]/instillate ^{125}I-albumin ratio = 0.85 ± 0.04, mean ± SD). Thus, the initial dilution of the labeled alveolar albumin tracer was secondary to dilution by protein-rich alveolar edema fluid already present in the alveoli. Because of the dilution of alveolar tracer to 85%, we assumed that the initial ^{125}I-albumin concentration was 85% of the instilled concentration. In addition, because 10.7% of the alveolar tracer appeared in the plasma, this quantity was subtracted from the measured alveolar counts as described in METHODS. Alveolar liquid clearance from the labeled ^{125}I-albumin data was then calculated with these two corrections.

The final-to-instilled unlabeled alveolar protein concentration ratio in acid-instilled rabbits did not increase as much as in control rabbits (Figure 3A). This corresponded to a markedly reduced rate of alveolar liquid clearance in acid instilled rabbits compared with the saline-instilled-rabbits, as measured by the concentration of unlabeled protein (Figure 4A). Similarly, the final-to-instilled labeled alveolar protein tracer concentration ratio (^{125}I-albumin ratio) in acid-instilled rabbits was lower than in control/saline-instilled rabbits (1.26 ± 0.30 versus 1.52 ± 0.40, $p < 0.05$). Thus, using the labeled alveolar protein tracer data, alveolar liquid clearance was significantly reduced in acid-instilled rabbits compared with saline-instilled rabbits (28 ± 3 versus 46 ± 4%, $p < 0.05$).

Effects of acid instillation on lung vascular barrier permeability. Total extravascular plasma equivalents were signifi-

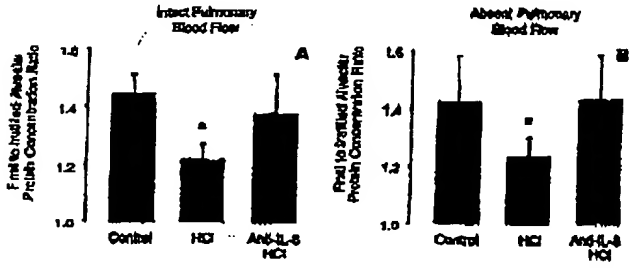

Figure 3. Ratio of the final-to-instilled unlabeled alveolar protein concentration measured in rabbits with intact pulmonary blood flow and in rabbits without vascular filtration. *$p < 0.05$ from saline-instilled rabbits, #$p < 0.05$ from acid-instilled rabbits. Data are mean ± SD.

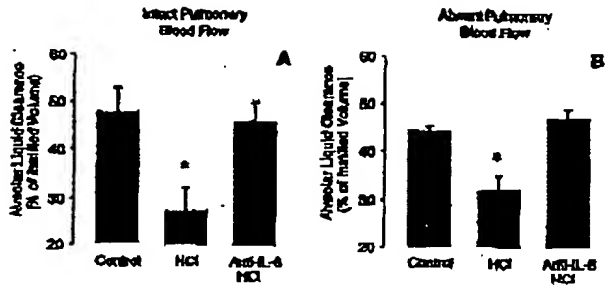

Figure 4. Alveolar liquid clearance (percent of instilled liquid) was measured by the increase of the unlabeled alveolar protein concentration in rabbits with intact pulmonary blood flow and in rabbits without vascular filtration. $^{*}p < 0.05$ from saline-instilled rabbits, $^{\#}p < 0.05$ from acid-instilled rabbits. Data are mean ± SD.

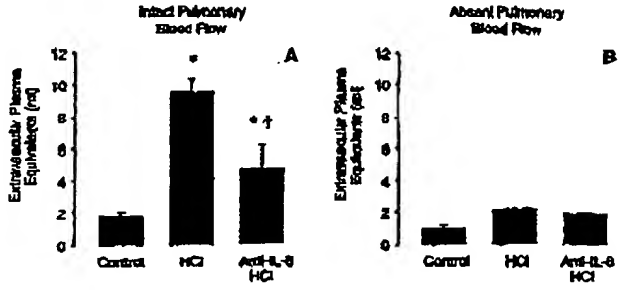

Figure 5. Lung endothelial permeability to protein measured as the accumulation of the vascular protein tracer, ^{131}I-albumin, in the extravascular space of the lung and expressed as extravascular plasma equivalents in rabbits with normal vascular filtration and in rabbits without vascular filtration. $^{*}p < 0.05$ from saline-instilled rabbits, $^{\#}p < 0.05$ from acid-instilled rabbits.

cantly increased in rabbits instilled with HCl compared with control rabbits instilled with saline alone (Figure 5A).

Anti-IL-8 pretreatment. Anti-IL-8 pretreatment significantly decreased bidirectional protein flux–induced acid aspiration. Pretreatment with the IL-8 antibody significantly decreased the permeability of the alveolar epithelial barrier to the alveolar tracer, ^{125}I-albumin, in acid-instilled rabbits (Table 1). There was also a significant decrease in the protein flux (vascular protein tracer, ^{131}I-albumin) from the plasma into the airspaces in the anti-IL-8-pretreated acid-instilled rabbits compared with acid-instilled rabbits (Table 1).

The final-to-instilled unlabeled alveolar protein concentration in acid-instilled rabbits that were pretreated with the anti-IL-8 antibody was significantly higher than in rabbits instilled with the acid alone (Figure 3A). Alveolar liquid clearance measured by the concentration of unlabeled protein was also significantly higher in the anti-IL-8-pretreated acid-instilled rabbits compared with acid-instilled rabbits (Figure 4A). Similarly, there was a significant increase in the final-to-instilled labeled alveolar protein tracer concentration ratio (^{125}I-albumin ratio) in anti-IL-8-pretreated acid-instilled rabbits compared with acid-instilled rabbits (1.46 ± 0.30 versus 1.26 ± 0.30, $p < 0.05$). There was no dilution of the radiolabeled albumin in these studies in the alveolar sample taken 5 min after instillation of the alveolar test solution. Thus, pretreatment with IL-8 antibody completely prevented a decrease in alveolar liquid clearance in acid-instilled rabbits (46 ± 4% HCl + anti-IL-8 versus 28 ± 3% HCl, $p < 0.05$).

Finally, pretreatment with IL-8 antibody significantly reduced the accumulation of extravascular plasma equivalents in acid-instilled rabbits (Figure 5A).

Studies without Pulmonary Blood Flow

Effects of acid instillation on alveolar epithelial permeability to protein. Despite the absence of pulmonary blood flow to the right lung, there was a small but significant increase in the permeability of the alveolar protein tracer, ^{125}I-albumin, across the lung epithelial barrier into the plasma in acid-instilled rabbits compared with saline-instilled rabbits (Table 1), perhaps related to the removal of ^{125}I-albumin by lung lymphatics and the bronchial circulation. However, there was no change in the alveolar-to-plasma ratio of the vascular protein tracer, ^{131}I-albumin, between the experimental groups (Table 1).

Effects of acid instillation on fluid transport across the alveolar barrier. Eliminating pulmonary blood flow had no effect on the acid-induced reduction in alveolar liquid clearance, as shown in Figures 3B and 4B.

In the absence of pulmonary blood flow, the effects of acid and anti-IL-8 on the alveolar epithelium could be assessed directly. There was a similar significant increase in the final-to-instilled unlabeled alveolar protein concentration ratio in the anti-IL-8-pretreated acid-instilled rabbits as in the rabbits with intact pulmonary blood flow (Figure 3B). Thus, alveolar liquid clearance as measured by the concentration of unlabeled protein was significantly increased in anti-IL-8-pretreated acid-instilled pretreated rabbits compared with untreated acid-instilled rabbits (Figure 4B). The final-to-instilled labeled alveolar protein tracer concentration ratio (^{125}I-albumin ratio) in the anti-IL-8-pretreated acid-instilled rabbits was significantly higher compared with acid-instilled rabbits that were not pretreated with anti-IL-8 (1.48 ± 1.00 versus 1.26 ± 0.30, $p < 0.05$). There was no dilution of the instilled radiolabeled tracer protein in any of the experiments without blood flow. Alveolar liquid clearance as measured by the increase of labeled alveolar protein tracer, ^{125}I albumin, was normal in the anti-IL-8-pretreated acid-instilled rabbits compared with acid-instilled rabbits that were not pretreated with anti-IL-8 (47 ± 1% versus 33 ± 2%, $p < 0.05$).

Effects of acid instillation on lung vascular barrier function. As expected, because of the absence of pulmonary blood flow, total extravascular plasma equivalents in the lung in rabbits instilled with hydrochloric acid were not significantly different from those measured in rabbits instilled with saline alone (Figure 5B). As observed in rabbits with normal pulmonary blood flow, pretreatment with the IL-8 antibody significantly reduced the permeability to the alveolar protein tracer, ^{125}I-albumin, in acid-instilled rabbits compared with acid-instilled rabbits that were not pretreated with IL-8 antibody. There was a significant decrease in the ^{125}I-albumin concentration in the plasma in the anti-IL-8-pretreated acid instilled rabbits compared with acid-instilled rabbits that were not pretreated with anti-IL-8 (Table 1).

DISCUSSION

The overall objective of these studies was to investigate the function of the alveolar epithelial barrier after acid-induced lung injury and whether damage could be prevented by cytokine blockade. Because acid aspiration lung injury is a major cause of acute lung injury in humans, several experimental (6–9, 11, 12) and clinical (1, 2, 24–26) studies have been carried out to determine the mechanisms that mediate the acute lung injury. However, most of these studies have not addressed the effect of acid aspiration on the function of the alveolar epithelium. In contrast to our prior studies of acid instillation lung

injury which focused on lung endothelial injury (7, 8), this study was designed specifically to measure alveolar epithelial barrier function and fluid transport capacity after acid-induced lung injury.

Previous studies from our laboratory reported that acid aspiration with a pH of 1.5 causes severe lung endothelial injury with the development of pulmonary edema (7, 8). Acid aspiration–induced lung injury is mediated primarily by activated neutrophils recruited to the lung by proximal acid-induced cytokines (4, 7–9, 11, 27). Acid aspiration results in a high concentration of IL-8 and large numbers of neutrophils in the airspaces (7). IL-8 is the major chemotactic cytokine that recruits neutrophils to the injured lung (28, 27). Anti-IL-8, applied as either pretreatment or treatment, decreased the magnitude of acute lung injury as measured by improved oxygenation, lower extravascular lung water, and a reduction in transvascular protein permeability (7). However, the specific effect of anti-IL-8 therapy on the function of the alveolar epithelium after acid instillation, either in terms of protein permeability or the alveolar fluid transport capacity, was not known. Therefore, these experiments were designed to evaluate the hypothesis that anti-IL-8 therapy would reduce alveolar epithelial injury.

Studies of the alveolar epithelium are potentially complicated by effects on the pulmonary endothelium. In our prior studies of alveolar fluid clearance in the presence of either endotoxemia or bacteremia, a moderate increase in lung endothelial permeability to protein did not diminish the fluid transport capacity of the alveolar epithelium (16). However, if the lung endothelial injury is severe, then a large transvascular filtration can prevent any net alveolar fluid clearance, as we found in the early phase of severe oleic acid–induced lung injury in sheep (15). Therefore, in these rabbit studies, we carried out experiments in which pulmonary blood flow was eliminated to avoid the potential confounding effects of increased lung vascular filtration on alveolar epithelial fluid transport. Interestingly, the studies without vascular filtration indicated that acid-induced lung injury still resulted in a decreased transport capacity of the alveolar epithelium to approximately 50% of normal levels (Figures 5A and 5B).

The method for measuring alveolar fluid clearance in the presence of injury has been validated in our prior studies (22, 23). We calculated the dilution of the ^{125}I-albumin in samples obtained 5 min after the beginning of the instillation (85% of instilled). We also adjusted for the 10.7% loss of the alveolar tracer over 2 h into the plasma. With these two adjustments, alveolar liquid clearance was calculated. The results seem internally consistent because calculation of alveolar liquid clearance based on the adjusted ^{125}I-albumin data in the acid-injured lungs was similar to the calculation of alveolar liquid clearance with unlabeled protein. Why was there a good agreement between the two methods? With the unlabeled protein method, the instilled protein concentration (5% albumin) was probably similar to the protein concentration of the edema fluid already present in the airspaces. Secondly, although approximately 10 to 11% of the unlabeled protein was probably lost from the airspaces (as was the case for the ^{125}I-albumin), it is likely that an approximately equivalent quantity of unlabeled plasma protein exuded into the airspaces. There is evidence that some of the vascular tracer, ^{131}I-albumin, accumulated in the airspaces (Table 1). Thus, the concentration of the unlabeled total protein over 2 h was proportional to the concentration of the ^{125}I-albumin over 2 h when the ^{125}I-albumin was adjusted for the initial dilution of alveolar edema fluid and the 10.7% loss into plasma over 2 h. As additional support for the validity of these calculations of alveolar liquid clearance, alveolar liquid clearance was similar in both the untreated acid-injured rabbits and the anti-IL-8–pretreated rabbits in the presence or absence of pulmonary blood flow and vascular filtration (Figure 4).

It is remarkable that alveolar epithelial fluid transport capacity continued at approximately 50% of normal levels after a low pH acid-induced lung injury. This result confirms that despite significant injury to the epithelial barrier, some level of alveolar epithelial fluid transport persists. Most patients with clinical acute lung injury who develop severe alveolar edema must reach a steady state in which fluid formation approximately balances fluid clearance. If this were not the case, the lung would be overwhelmed with alveolar edema fluid. In other words, alveolar flooding markedly impairs gas exchange, but the preservation of a reduced level of alveolar fluid transport coupled with lung lymphatic clearance probably allows the injured lung to reach a steady state in lung fluid balance, albeit in the presence of significant alveolar and interstitial pulmonary edema.

Several previous studies have demonstrated that acid aspiration–induced lung injury is mediated in part by neutrophils (4, 7–9, 11, 27). In our prior study of acid-induced lung injury, the number of neutrophils in the airspaces was reduced by 50% at 6 h and by 80% at 24 h after the injury when anti-IL-8 was given either as pretreatment or as treatment (7). It is likely therefore that the protective effect of anti-IL-8 therapy on the alveolar epithelium was related to the inhibitory effect on neutrophil migration into the airspaces of the lung.

There are some limitations to this study. First, we did not carry out both treatment and pretreatment studies, unlike our prior anti-IL-8 study in rabbits with acid-induced lung injury (7). However, our prior study demonstrated that the same anti-IL-8 monoclonal antibody that was used in this study was efficacious when administered 1 h after acid instillation in preventing 80% of the acid-induced lung injury; also the effect in that study was sustained for 24 h (7). The purpose of the current study was focused on the alveolar epithelium, first to determine the magnitude of functional injury from low pH acid to the epithelial barrier properties and epithelial fluid transporting capacity, and second to determine if the injury was mediated by IL-8–dependent mechanisms. Because the anti-IL-8 pretreatment prevented most of the epithelial injury, the evidence seems clear that IL-8, probably by neutrophil-dependent mechanisms, plays an important role in mediating acid-induced epithelial injury. Unlike some of our prior studies, we did not carry out histologic studies because we thought the physiologic studies would be better able to quantify the magnitude of epithelial injury. These studies were 6 h in duration, and therefore we cannot comment on the long-term effects of acid injury on the alveolar epithelial barrier. One might question the use of rabbits because, in contrast to the human lung (28), rabbits do not upregulate alveolar fluid clearance in response to β-adrenergic stimulation (29). Therefore, the direct relevance to the acid-injured human lung is uncertain. On the other hand, basal alveolar fluid clearance in rabbits is rapid, similar to intact alveolar fluid clearance based on *in vivo* studies of the resolution of alveolar edema in patients (19). Also, because rabbits do not respond to β-adrenergic agonists with an increase in alveolar fluid clearance, our measurements of alveolar fluid clearance under control or after acid-induced injury did not need to consider endogenous release of epinephrine, an important mechanism following septic or short-term hypovolemic shock in rats (30, 31). Finally, we did not treat the rabbits with a sham antibody, but this control seemed unnecessary, particularly since these controls were included in our prior acid instillation studies with this anti-IL-8 antibody in rabbits (7).

In summary, the results of this experimental study in rabbits indicate that alveolar epithelial fluid transport is reduced 4–6 h after acid-induced lung injury by approximately 50%. Neutralization of IL-8 before acid instillation prevents alveolar epithelial injury, as measured by both alveolar epithelial permeability to protein and alveolar epithelial fluid transport capacity. These results provide further support for the possible clinical value of anti-IL-8 therapy from aspiration of low pH gastric contents, as was suggested by our prior rabbit study (7).

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References

1. Pepe, P. E., R. T. Potkin, D. H. Reus, C. D. Hudson, and C. J. Carrico. 1982. Clinical predictions of the adult respiratory distress syndrome. *Am. J. Surg.* 144:124–130.
2. Fowler, A. A., III, R. F. Hamman, and J. T. Good. 1983. Adult respiratory distress syndrome: risk with common predispositions. *Ann. Intern. Med.* 98:593–597.
3. Goldman, G., R. Welbourn, J. M. Klausner, I. S. Paterson, L. Kobzik, C. R. Valeri, D. Shepro, and H. B. Hechtman. 1989. Localized acid aspiration leads to thromboxane dependent generalized pulmonary edema. *Surg. Forum* 40:258–260.
4. Kennedy, T. P., K. J. Johnson, R. G. Kunkel, P. A. Ward, P. R. Knight, and J. S. Finch. 1989. Acute acid aspiration lung injury in the rat: biphasic pathogenesis. *Anesth. Analg.* 69:87–92.
5. Goldman, G., R. Welbourn, L. Kobzik, C. R. Valeri, D. Shepro, and H. B. Hechtman. 1992. Reactive oxygen species and elastase mediate lung permeability after acid aspiration. *J. Appl. Physiol.* 73:571–575.
6. St. John, R. C., L. A. Miller, G. C. Kindt, S. R. Weisbrode, S. A. Moore, and P. M. Dorinsky. 1993. Acid aspiration-induced acute lung injury causes leukocyte-dependent systemic organ injury. *J. Appl. Physiol.* 74:1994–2003.
7. Folkesson, H. G., M. A. Matthay, C. A. Hébert, and V. C. Broaddus. 1995. Acid aspiration-induced lung injury in rabbits is mediated by interleukin-8-dependent mechanisms. *J. Clin. Invest.* 96:107–116.
8. Folkesson, H. G., and M. A. Matthay. 1997. Inhibition of CD18 or CD11b attenuates acute lung injury after acid instillation in rabbits. *J. Appl. Physiol.* 82:1743–1750.
9. Ishii, Y., J. Kobayashi, and S. Kitamura. 1989. Chemotactic factor generation and cell accumulation in acute lung injury induced by endotracheal acid instillation. *Prostaglandins Leukot. Essent. Fatty Acids* 37: 65–70.
10. Doerschuk, C. M., R. K. Winn, H. O. Coxson, and J. M. Harlan. 1990. CD18-dependent and -independent mechanisms of neutrophil emigration in the pulmonary and systemic microcirculation of rabbits. *J. Immunol.* 144:2327–2333.
11. Goldman, G., R. Welbourn, L. Kobzik, C. R. Valeri, D. Shepro, and H. B. Hechtman. 1990. Tumor necrosis factor-α mediates acid aspiration-induced systemic organ injury. *Ann. Surg.* 212:513–520.
12. Goldman, G., R. Welbourn, J. M. Klausner, L. Kobzik, C. R. Valeri, D. Shepro, and H. B. Hechtman. 1991. Neutrophil accumulation due to pulmonary thromboxane synthesis mediate acid aspiration injury. *J. Appl. Physiol.* 70:1511–1517.
13. Miller, E. J., A. B. Cohen, S. Nagao, D. Griffith, R. J. Maunder, T. R. Martin, J. P. Weiner-Kronish, M. Sticherling, E. Christophers, and M. A. Matthay. 1992. Elevated levels of NAP-1/interleukin-8 are present in the airspaces of patients with the adult respiratory distress syndrome and are associated with increased mortality. *Am. Rev. Respir. Dis.* 146:427–432.
14. Donnelly, S. C., R. M. Strieter, S. L. Kunkel, A. Walz, C. R. Robertson, D. C. Carter, I. S. Grant, A. J. Pollok, and C. Haslett. 1995. Interleukin-8 and development of adult respiratory distress syndrome in at-risk patient groups. *Lancet* 341:643–647.
15. Wiener-Kronish, J. P., V. C. Broaddus, K. H. Albertine, M. A. Gropper, M. A. Matthay, and N. C. Staub. 1988. Relationship of pleural effusions to increased permeability pulmonary edema in anesthetized sheep. *J. Clin. Invest.* 82:1422–1429.
16. Wiener-Kronish, J. P., K. H. Albertine, and M. A. Matthay. 1991. Differential responses of the endothelial and epithelial barriers of the lung in sheep to *Escherichia coli* endotoxin. *J. Clin. Invest.* 88:864–875.
17. Pittet, J. F., J. P. Wiener-Kronish, V. Serikov, and M. A. Matthay. 1995. Resistance of the alveolar epithelium to injury from septic shock in sheep. *Am. J. Respir. Crit. Care Med.* 151:1093–1100.
18. Folkesson, H. G., G. Nitenberg, B. L. Oliver, C. Jayr, K. H. Albertine, and M. A. Matthay. 1998. Upregulation of alveolar epithelial fluid transport after subacute lung injury in rats from bleomycin. *Am. J. Physiol.* 275:L478–L490.
19. Matthay, M. A., and J. P. Wiener-Kronish. 1990. Intact epithelial barrier function is critical for the resolution of alveolar edema in humans. *Am. Rev. Respir. Dis.* 142:1250–1257.
20. Broaddus, V. C., A. M. Boylan, J. M. Hoeffel, K. J. Kim, M. Suttick, A. Chantharaphol, and C. A. Hébert. 1994. Neutralization of interleukin-8 inhibits neutrophil influx in a rabbit model of endotoxin-induced pleurisy. *J. Immunol.* 152:2960–2967.
21. Berthiaume, Y., N. C. Staub, and M. A. Matthay. 1987. Beta-adrenergic agonists increase lung liquid clearance in anesthetized sheep. *J. Clin. Invest.* 79:335–343.
22. Folkesson, H. G., F. Kheradmand, and M. A. Matthay. 1994. The effect of salt water on alveolar epithelial barrier function. *Am. J. Respir. Crit. Care Med.* 150:1555–1563.
23. Rezaiguia, S., C. Garat, C. Delclaux, M. Meignan, J. Fleury, P. Legrand, M. A. Matthay, and C. Jayr. 1997. Acute bacterial pneumonia in rats increases alveolar epithelial fluid clearance by a tumor necrosis factor-alpha-dependent mechanism. *J. Clin. Invest.* 99:325–335.
24. Awe, W. C., W. S. Fletcher, and S. W. Jacob. 1966. The pathophysiology of aspiration pneumonitis. *Surgery* 60:232–236.
25. Bynum, L. J., and A. K. Pierce. 1976. Pulmonary aspiration of gastric contents. *Am. Rev. Respir. Dis.* 114:1129–1136.
26. Donnelly, S. C., R. M. Strieter, S. L. Kunkel, A. Walz, D. Steedman, I. S. Grant, A. J. Pollok, D. C. Carter, and C. Haslett. 1994. Chemotactic cytokines in the established adult respiratory distress syndrome and at-risk patients. *Chest* 105:985–993.
27. Goldman, G., R. Welbourn, J. M. Klausner, L. Kobzik, C. R. Valeri, D. Shepro, and H. B. Hechtman. 1993. Leukocytes mediate acid aspiration-induced multiorgan edema. *Surgery* 114:12–20.
28. Sakuma, T., H. G. Folkesson, S. Suzuki, G. Okaniwa, S. Fujimura, and M. A. Matthay. 1997. Beta-adrenergic agonist stimulated alveolar fluid clearance in *ex vivo* human and rat lungs. *Am. J. Respir. Crit. Care Med.* 155:505–512.
29. Smedira, N., L. Gates, R. Hastings, C. Jayr, T. Sakuma, J. F. Pittet, and M. A. Matthay. 1991. Alveolar liquid clearance in anesthetized rabbits. *J. Appl. Physiol.* 70:1827–1836.
30. Pittet, J. F., J. P. Wiener-Kronish, M. C. McElroy, H. G. Folkesson, and M. A. Matthay. 1994. Stimulation of alveolar epithelial liquid clearance by endogenous release of catecholamines in septic shock. *J. Clin. Invest.* 94:663–671.
31. Pittet, J. F., T. J. Brenner, K. Modelska, and M. A. Matthay. 1996. Alveolar liquid clearance is increased by endogenous catecholamines in hemorrhagic shock in rats. *J. Appl. Physiol.* 81:830–837.